UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF HUMAN ENTEROVIRUS 71 NANO CALCIUM PHOSPHATE ADJUVANTED CANDIDATE VACCINE FOR PARENTERAL AND MUCOSAL DELIVERY

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By

MOHAMED IBRAHIM SAEED AHMED

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

This effort is dedicated to my parents, my wife, Basil and my family.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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A successful potent vaccine for protection against the faecal-oral pathogen human Enterovirus 71, requires the induction of both parenteral and mucosal immunity concurrently. Adsorbing the vaccine to a nano-sized particulate adjuvant will boost the systemic immune response towards parenteral administrated vaccine, and combining the vaccine adjuvant and delivery carrier in one formulation for delivering mucosal vaccines is expected to improve the post-vaccination HEV71 mucosal protection. The purpose of this work was to examine the applicability of inducing improved post-vaccination immunity of Human Enterovirus 71 killed vaccine based on using nano-sized vaccine-adjuvant particles, variable routes of parenteral immunization and different polymeric delivery carriers for inducing enhanced post vaccination immunity as a novel approach for the development of an effective HEV71 vaccine.

A novel *in vitro* delivery system was designed and used to examine the *in vitro* release of the HEV71 killed-virus and calcium phosphate (CaP) adjuvants from the encapsulating delivery carrier comprising nano-sized chitosan and micro-sized alginate hydrogels prepared and used to compare their *in vitro* capacity of releasing the vaccine and adjuvant using commercial HEV71-VP1 kit and a Calcium Calorimetric Quantification Kit. In addition, an intervention animal study was conducted on laboratory experimental rabbits to examine its capacity for inducing post-vaccination systemic IgG levels from HEV71 killed-virus adsorbed in nano- and micro-sized CaP-adjuvants through intradermal and intramuscular routes. Buccal delivery consisted of HEV71 killed-virus adsorbed with CaP-adjuvant encapsulated in chitosan and alginate hydrogels and with the unvaccinated group kept as a control. The animals were immunized with HEV71 vaccine formulation and blood samples were withdrawn at 0, 1, 3, 5 and 7 weeks post immunization, the last samples were collected two weeks after the last dose. The samples were examined for the presence of HEV71 specific IgG and IgA antibody classes in the serum and saliva using an in-house developed ELISA assay.

The *in vitro* delivery of adjuvant loaded polymeric carrier offered an improved and sustained release of the nano-adjuvant encapsulated in chitosan but not alginate, due to the reversibility of adjuvant-polymer interaction. Moreover, chitosan hydrogel showed better encapsulation and reversible interaction with both the nano- and micro-sized adjuvant particles. The *in vitro* delivery of HEV71 killed-virus adsorbed adjuvant loaded onto a polymeric carrier displayed a superior capacity for generating an optimized sustained release of vaccine epitopes. Chitosan loaded nano-adjuvant offered ascending and extended vaccine antigen release, due to its smaller adjuvant size and reversibility of interaction, but not in alginate-adjuvant formulations, due the vaccine been sequestered in the calcium-alginate chemical crosslink.

In immunized animals, the nano-CaP in a one-tenth millilitre intradermal dose induced a higher level of viral specific antibody levels, which was almost equal to the intramuscular one millilitre administered after five doses. The nano-sized CaP was capable of producing a higher level of viral specific antibodies compared to the micro-particle sized commercial adjuvant or the vaccine without adjuvant when administered through the intradermal route. Intramuscular immunization with either nano- or micro-particles CaP adsorbed HEV71 killed-virus was able to induce higher viral specific antibodies than the nano-CaP adsorbed vaccine alone.

Chitosan encapsulation of the vaccine or a vaccine with nano-CaP induced a high IgA level due to its extended swelling, and reversibility of releasing the vaccine. The alginate formulations had no effect on the IgA levels, it seems the vaccine epitopes were trapped in the alginate composite. The effect of joint dual immunization parenteral and mucosal routes (nano-CaP-HEV71 killed-virus (ID) and vaccine encapsulated in chitosan through the buccal delivery) to the same animals induced enhanced both types of systemic and mucosal antibody responses, as displayed in serum IgG and salivary IgA antibody levels towards the vaccine. The vaccine being adsorbed into the nano-CaP (intradermal) offered enhanced virus neutralization in animal serum samples. However, the enhancement of the virus neutralization in saliva, seemed to be hindered by the flow of the salivary secretion.

In conclusion, the application of a novel nano-sized calcium phosphate as adjuvant is a promising strategic approach for the development of an improved HEV71 post-vaccination parenteral immunity. In addition, polymeric carrier concentration, hydrogel swelling capacity, the nature of adjuvant-carrier interaction, are important factors for the sustained release process of CaP-adjuvant and HEV71 killed-virus on the novel created vaccine delivery model. The use of a nano-sized adjuvant encapsulated in a muco-adhesive carrier introduces a novel system for the enhancement of post vaccination mucosal immunity not only through the buccal mucosa, but also to the other mucosal surfaces, such as nasal or urogenital.

Furthermore, the joint vaccine administration through the parenteral and mucosal routes enhanced the level of secretory IgA antibody in the saliva. In addition, an elevated post vaccination humoral immune response of a highly specific IgG and neutralizing antibodies was as shown in the *in vitro* inhibition of HEV71 live virus infectivity to Vero cell at the highest sample dilutions. Finally, the study finding is a key indicator for the role of nano-size adjuvant and carriers in augmenting the post-vaccination immunity. This could be applied to improve the post-vaccination immunity against other medical pathogens without limiting to Human Enterovirus 71.

Abstrak tesis ini dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi syarat keperluan untuk ijazah Doktor Falsafah

PEMBANGUNAN MANUSIA ENTEROVIRUS 71 NANO KALSIUM FOSFAT ADJUVANTED CALON VAKSIN UNTUK PENGHANTARAN PARENTERAL DAN LAMBUNG

Oleh

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Kerja-kerja ini bertujuan untuk mengkaji kebolehgunaan mendorong lebih baik imuniti selepas vaksinasi terhadap manusia Enterovirus-71 terbunuh vaksin berdasarkan menggunakan zarah pembolehubah bersaiz laluan parenteral ubah vaksin yg membantu imunisasi dan menggunakan pembawa polimer yang berbeza untuk mendorong penghantaran yang dipertingkatkan pos imuniti vaksinasi sebagai pendekatan baru untuk vaksin yang berkesan membangunkan HEV-71.

Sistem penyampaian dalam vitro novel direka dan digunakan untuk memeriksa pelepasan dalam vitro dibunuh HEV71-virus dan kalsium fosfat (CaP) adjuvants daripada syarikat pengangkutan penghantaran encapsulating yang terdiri daripada chitosan bersaiz nano dan hydrogels alginate bersaiz mikro disediakan dan digunakan untuk membandingkan kapasiti mereka dalam vitro melepaskan pelalian dan menormalkan menggunakan kit HEV71-VP1 komersial dan Kit mengkuantifikasi Calorimetric kalsium.

Penambahan haiwan dalam kajian campur tangan dalam arnab makmal eksperimen dijalankan untuk memeriksa kapasitinya untuk menggalakkan tahap IgG sistemik selepas suntikan daripada HEV71 vaksin adsorbed dalam nano dan mikro-bersaiz CaP-adjuvants melalui laluan intradermal. Penghantaran buccal terdiri daripada HEV71 membunuh virus adsorbed dengan CaP-menormalkan dimuatkan ke dalam hydrogels chitosan dan alginate dan Kumpulan unvaccinated yang disimpan sebagai kawalan.

Haiwan imunisasi dengan vaksin HEV71 formulasi dan sampel darah telah ditarik balik selepas pelalian pada minggu 0, 1, 3, 5 dan 7, mengumpul sampel dua minggu lepas selepas dos yang lepas. Sampel kajian telah disemak untuk kehadiran khusus IgG dan IgA kelas antibodi HEV71 serum dan air liur yang menggunakan cerakin ELISA yang maju.

Adsorbing pelalian untuk menormalkan zarah bersaiz nano akan meningkatkan respon imun sistemik terhadap vaksin berpandu parenteral, dan menggabungkan vaksin menormalkan dan penghantaran pembawa dalam satu formulasi untuk menyampaikan lambung vaksin dijangka bertambah baik selepas suntikan HEV71 lambung perlindungan.

Penghantaran dalam vitro menormalkan dimuatkan di fromula pembawa chitosan ditawarkan siaran nano-menormalkan yang dimuatkan ke dalam chitosan tetapi tidak alginate yang lebih baik dan mampan. ini disebabkan oleh irreversibility kalsium-alginate interaksi.

Haiwan mendapat imunisasi, nano-topi dalam yang dos intradermal satu persepuluh mililiter induced tahap yang lebih tinggi tahap antibodi virus tertentu, bersamaan dengan intramuskular jumlah dos satu mililiter. Topi bersaiz nano adalah mampu menghasilkan tahap yang lebih tinggi daripada virus antibodi tertentu berbanding dengan micro-zarah yang bersaiz menormalkan atau pelalian tanpa menormalkan melalui laluan intradermal.

Imunisasi dengan mana-mana nano - atau mikro-zarah CaP adsorbed HEV71 membunuh virus telah berjaya memujuk lebih tinggi virus tertentu antibodi daripada nano-CaP adsorbed vaksin sahaja. Chitosan encapsulation pelalian atau vaksin dengan nano-CaP induced tahap IgA yang tinggi berikutan lanjutan bengkak, dan keterbalikan melepaskan pelalian. Formulasi alginate mempunyai kesan ke atas tahap IgA, ia seolah-olah epitopes vaksin terkandas dalam komposit alginate.

Kesan pelalian dwi bersama parenteral dan lambung laluan (nano-CaP-HEV71 membunuh virus (ID) dan vaksin yang dimuatkan ke dalam chitosan melalui penyampaian buccal) terhadap haiwan-haiwan yang sama induced dipertingkatkan kedua-dua jenis tindakbalas antibodi sistemik dan lambung, seperti yang dipaparkan dalam serum IgG dan tahap antibodi IgA salivary ke arah pelalian.

Kesimpulannya, penggunaan novel yang bersaiz-nano fosfat kalsium menormalkan adalah pendekatan strategik yang menjanjikan untuk pembangunan yang lebih baik HEV71 selepas suntikan parenteral imuniti. Di samping itu, kepekatan polymeric pembawa, hydrogel bengkak kapasiti, jenis interaksi menormalkan-pembawa, adalah faktor yang penting untuk proses pelepasan berterusan CaP-menormalkan dan HEV71 dibunuh-virus pada model penghantaran novel vaksin yang dicipta.

Penggunaan menormalkan bersaiz nano yang dimuatkan ke dalam sebuah pesawat yang muco-pelekat memperkenalkan satu sistem baru untuk meningkatkan jawatan suntikan lambung imuniti bukan sahaja melalui pihak buccal mucosa, tetapi juga untuk di lambung permukaan lain, seperti saluran hidung atau urogenital.

Selain itu, pentadbiran bersama vaksin melalui laluan parenteral dan lambung dipertingkatkan tahap antibodi IgA secretory di dalam air liur. Di samping itu, bertingkat untuk menghantar suntikan response imun perubatan daripada IgG yang sangat khusus dan meneutralkan antibodi adalah seperti yang ditunjukkan dalam kesan infectivity virus hidup HEV71 ke Vero sel pada dilutions contoh yang tertinggi di dalam vitro.

Akhir sekali, dapatan kajian adalah penunjuk utama bagi peranan menormalkan bersaiz nano dan pembawa peningkatan kekebalan selepas suntikan. Ini boleh digunakan untuk meningkatkan kekebalan selepas imunisasi terhadap patogen lain perubatan tanpa menghadkan kepada manusia Enterovirus 71.

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Mohamed Ibrahim Saeed

This thesis was submitted to the senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

Alg	Alginate.
APC	Antigen-presenting cells
BALT	Bronchus-associated lymphoid tissue
BSA	Bovine serum albumin
CaP	Calcium Phosphate.
CH	Chitosan.
CPE	Cytopathic effect
CV-16	Coxsackievirus-16
ELISA	Enzyme-linked immunosorbent assay
HEV71	Human enterovirus 71
HFMD	Hand, Foot and Mouth disease
HRP	Horseradish peroxidase
MOI	Multiplicity of infection
OPV	Oral Poliovirus Vaccine
PRNT	Plaque Reduction Neutralization Test
PV	Poliovirus
RD	Rhabdomyosarcoma
RPMI	Roswell Park Memorial Institute medium
TMB	Teta methyle benzidine
TCID50	50% of Tissue Culture Infective Dose
VERO	African Green Monkey Kideny
VP	Viral structural protein
FTIR	Fourier transform infrared spectroscope
TEM	Transmission electron microscope
FESEM	Field Emission Scanning Electron Microscope
C.H.N.S	Carbon Hydrogen Nitrogen and Sulpher

CHAPTER 1

INTRODUCTION

Vaccination is the most important tool for preventing infectious diseases. Vaccines are the biological preparations of the immune-protective microbial antigen (s). It ' s commonly used as a key prevention tool against viral infections such as poliovirus (Attenweiler & Thomure, 2014). They are the only accessible pre-exposure applicable preventive means against Human Enterovirus 71 (HEV71), it was identified in 1969 as a cause agent of Human Hand, Foot and Mouth Disease (HFMD), a viral disease that attacks children of school age. The virus became among the most common pandemic disease after poliomyelitis (Debing, Jochmans, & Neyts, 2013). East Asia is the largest endemic region in which HEV71 infection spreads and the most affected countries include China, Japan, Taiwan, Malaysia, Singapore, Cambodia and Indonesia (Ma *et al*., 2009: Jing *et al*., 2012). Therefore, it became a necessity to develop an effective broad protective HEV71 vaccine. Antivirals and preventive vaccine, are commercially unavailable yet (Yu *et al*., 2007; Sarma, 2013). The enhancement of mucosal immunity is crucial due to the mode of virus transmission and shedding portals. And mucosal immunity plays an important role in the prevention of the mucosal associated infectious diseases and for that a mucosal delivery of vaccines has become a top demand, and the use of vaccine adjuvants or delivery carriers, seems the best available approach to induce a measurable mucosal immunity.

The main goal of the study was to develop an improved viral-specific systemic and mucosal post-vaccination immune responses toward HEV-71, through novel vaccine designs and formulations based on the routes of vaccine administration.

1.1 Problem Statement:

In general, faecal-oral pathogen, such as HEV71, requires the induction of both parenteral and mucosal immunity concurrently. This needs to be achieved in line with the assurance of avoiding vaccine strain-derived infections.

Therefore the approach followed in this study involve the use of A nano-size, non-toxic and bio-compatible substance (calcium-phosphate) was chosen for preparing the adjuvant and biodegradable polymers was chosen as a carrier for the vaccine adsorbed on adjuvant as follows: First, adsorbing the vaccine to a nano-sized particulate adjuvant of an increased surface area compared to the conventionally used micro-particle or adjuvant of unknown size, which will delay and extend the release of the vaccine antigens. Then, administering the vaccine through intradermal route is again expected to offer a delayed release of un-adsorbed vaccine antigens. Moreover, the intradermal immunization with vaccine adsorbed to nano-size adjuvant is expected to have a synergistic role in controlling the vaccine release and extending its capacity to activate increased lymphocytes and elevate the systemic antibody towards the nano-adjuvanted killed HEV71 virus epitopes.

The polymeric carriers (chitosan & alginate) are expected to provide improved delivery and mucosal uptake of vaccine. This is due to its slow-swelling property which will

help to prolong the presentation of the vaccine, its muco-adhesion will improve the adherence of the vaccine to the mucosal surface and antigen trans-mucosal delivery by the M-cell and APCs. It will protect the protein-based vaccine offering an enhanced stability against the degrading enzymatic, acidity and elevated temperature on the mucosal linings. Therefore, the combined formula of particulate nano-size adjuvant encapsulated muco-adhesive carrier for vaccine delivery through the buccal mucosa, can offer a prolonged and enhanced post-vaccination HEV-71 mucosal protection.

In addition, the concurrent dual vaccination with vaccine adsorbed on nano-adjuvant particles combined with mucosal delivery of vaccine adsorbed adjuvant coated into muco-adhesive carrier is expected to provide an improved mucosal immune response (IgA). This is a novel strategy for HEV-71 and pathogens of the oral, respiratory or sexually transmitted infections.

1.1.1 Study aim:

The goal of this research work was the development of a safer, inactivated HEV-71 vaccine to be administered simultaneously through intradermal using a nano-sized adjuvant and orally using a novel vaccine delivery carrier formulations to concurrently develop combined systemic and mucosal immune responses.

1.2 Objectives of the study:

1.2.1 General objective:

To develop and evaluate Human enterovirus 71 nano- and micro-size calcium phosphate adjuvant adsorbed vaccine for parenteral administration and mucosal delivery of vaccine loaded carrier.

1.2.2 Specific objectives:

1.2.2.1 To examine the *in vitro* release of different calcium phosphate adjuvant particle size loading delivery carriers.

1.2.2.2 To examine the *in vitro* delivery of HEV71 killed-virus candidate vaccine from different formulations of two calcium phosphate adjuvants encapsulated in delivery polymeric carriers.

1.2.2.3 To evaluate the role of adjuvant particle size and administration routes on the HEV71 killed-virus systemic antibody response.

1.2.2.4 To compare the antibody response to mucosal delivery of HEV71 killed-virus encapsulated in different formulations of calcium phosphate adjuvant and polymeric carriers.

1.2.2.5 To compare the *in vitro* neutralizing antibody titres towards HEV71 killed-virus in animal samples post parenteral and mucosal immunization.

REFERENCES

Aguilar, J., & Rodriguez, E. (2007). Vaccine adjuvants revisited. *Vaccine, 25*(19), 3752-3762.

Alphs, H. H., Gambhira, R., Karanam, B., Roberts, J. N., Jagu, S., Schiller, J. T., Roden, R. B. (2008). Protection against heterologous human papillomavirus challenge by a synthetic lipopeptide vaccine containing a broadly cross-neutralizing epitope of L2. *Proceedings of the National Academy of Sciences, 105*(15), 5850-5855.

Andrade, F., Antunes, F., Vanessa Nascimento, A., Baptista da Silva, S., das Neves, J., Ferreira, D., & Sarmento, B. (2011). Chitosan formulations as carriers for therapeutic proteins. *Current Drug Discovery Technologies, 8*(3), 157-172.

Arita, M., Nagata, N., Iwata, N., Ami, Y., Suzaki, Y., Mizuta, K., Shimizu, H. (2007). An attenuated strain of enterovirus 71 belonging to genotype a showed a broad spectrum of antigenicity with attenuated neurovirulence in cynomolgus monkeys. *Journal of virology, 81*(17), 9386-9395.

Attenweiler, G., & Thomure, A. (2014). Best Practices: A Network Approach of the Mandatory Influenza Vaccination Among Healthcare Workers.

Baqar, S., Bourgeois, A. L., Schultheiss, P. J., Walker, R. I., Rollins, D. M., Haberberger, R. L., & Pavlovskis, O. R. (1995). Safety and immunogenicity of a prototype oral whole-cell killed< i> Campylobacter</i> vaccine administered with a mucosal adjuvant in non-human primates. *Vaccine, 13*(1), 22-28.

Barbara C. Baudner, Maurizio Morandi, Marzia M. Giuliani, J.Coos Verhoef, Hans E, Junginger, Paolo Costantino, Rino Rappuoli, and Giuseppe Del Giudice, (2004),Modulation of Immune Response to Group C Meningococcal Conjugate Vaccine Given Intranasally to Mice Together with the LTK63 Mucosal Adjuvant and the Trimethyl Chitosan Delivery System, *The Journal of Infectious Diseases*; 189:828–32.

Bek, E. J., Hussain, K. M., Phuektes, P., Kok, C. C., Gao, Q., Cai, F., McMinn, P. C. (2011). Formalin-inactivated vaccine provokes cross-protective immunity in a mouse model of human enterovirus 71 infection. *Vaccine, 29*(29), 4829-4838.

Belshe, R. B., Mendelman, P. M., Treanor, J., King, J., Gruber, W. C., Piedra, P., .Zangwill, K. (1998). The efficacy of live attenuated, cold-adapted, trivalent, intranasal influenzavirus vaccine in children. *New England Journal of Medicine, 338*(20), 1405-1412.

Bernstein, D. I., Smith, V. E., Sherwood, J. R., Schiff, G. M., Sander, D. S., DeFeudis, D., Ward, R. L. (1998). Safety and immunogenicity of live, attenuated human rotavirus vaccine 89-12. *Vaccine, 16*(4), 381-387.

Bindu M. Boddupalli, Zulkar N. K. Mohammed, Ravinder A. Nath, and David Banji, (2010), Mucoadhesive drug delivery system: An overview, *J Adv Pharm Technol Res*, 1(4): 381–387, doi: 10.4103/0110-5558.76436, PMCID: PMC3255397.

Baqar, S., Bourgeois, A. L., Schultheiss, P. J., Walker, R. I., Rollins, D. M., Haberberger, R. L., & Pavlovskis, O. R. (1995). Safety and immunogenicity of a prototype oral whole-cell killed< i> Campylobacter</i> vaccine administered with a mucosal adjuvant in non-human primates. *Vaccine*, *13*(1), 22-28.

Bitsaktsis, C., Wiedinger, K., & Gosselin, E. J. (2013). Targeting cellular receptors as a strategy for the development of new generation mucosal vaccines. *American Journal of Immunology*, *9*(1), 9.

Blomqvist, S., Klemola, P., Kaijalainen, S., Paananen, A., Simonen, M.-L., Vuorinen, T., & Roivainen, M. (2010). Co-circulation of coxsackieviruses A6 and A10 in hand, foot and mouth disease outbreak in Finland. *Journal of Clinical Virology*, *48*(1), 49-54.

Borges, O., Cordeiro-da-Silva, A., Romeijn, S. G., Amidi, M., de Sousa, A., Borchard, G., & Junginger, H. E. (2006). Uptake studies in rat Peyer's patches, cytotoxicity and release studies of Alginate coated Chitosan nanoparticles for mucosal vaccination. *Journal of controlled release*, *114*(3), 348-358.

Boursnell, M., Entwisle, C., Blakeley, D., Roberts, C., Duncan, I., Chisholm, S., Sobek, I. (1997). A genetically inactivated herpes simplex virus type 2 (HSV-2) vaccine provides effective protection against primary and recurrent HSV-2 disease. *Journal of Infectious Diseases*, *175*(1), 16-25.

Brennan, F. R., Bellaby, T., Helliwell, S. M., Jones, T. D., Kamstrup, S., Dalsgaard, K., Hamilton, W. D. (1999). Chimeric plant virus particles administered nasally or orally induce systemic and mucosal immune responses in mice. *Journal of virology*, *73*(2), 930-938.

Bruce, M. (2010). *DFID's performance in 2008-09 and the 2009 White Paper: fourth report of session 2009-10, Vol. 1: Report, together with formal minutes* (Vol. 1): TSO Shop.

Calvo P, Remunan-Lopez C, Vila-Jato JL, Alonso MJ. (1997) Novel hydrophilic Chitosan-polyethylene oxide nanoparticles as protein carriers. *J Appl Polym Sci*.; 63:125-32.

Cao, L., Yi, Y., Song, J., Tian, M., Tian, R., Meng, Q., Bi, S. (2012). The assemblage, purification and characterization of EV71 VLPs expressed in baculovirus]. *Bing du xue bao= Chinese journal of virology/[bian ji, Bing du xue bao bian ji wei yuan hui]*, *28*(3), 201.

Cao, Y.-G., Li, Z.-H., Yue, Y.-Y., Song, N.-N., Peng, L., Wang, L.-X., & Lu, X. (2013). Construction and evaluation of a novel Bacillus subtilis sporesbased enterovirus 71 vaccine. *Journal of Applied Biomedicine*, *11*(2), 105-113.

Caputo, A., Sparnacci, K., Ensoli, B., & Tondelli, L. (2008). Functional polymeric nano/microparticles for surface adsorption and delivery of protein and DNA vaccines. *Current drug delivery*, *5*(4), 230-242.

Chadwick, S., Kriegel, C., & Amiji, M. (2010). Nanotechnology solutions for mucosal immunization. *Advanced drug delivery reviews*, *62*(4), 394-407.

Chang, J.-Y., Chang, C.-P., Tsai, H. H.-P., Lee, C.-D., Lian, W.-C., Sai, I.-H, Chen, C.-Y. (2012). Selection and characterization of vaccine strain for Enterovirus 71 vaccine development. *Vaccine*, 30(4), 703-711.

Chan, L., Parashar, U. D., Lye, M., Ong, F., Zaki, S. R., Alexander, J. P., Suleiman, A. B. (2000). Deaths of children during an outbreak of hand, foot, and mouth disease in Sarawak, Malaysia: clinical and pathological characteristics of the disease. *Clinical infectious diseases*, *31*(3), 678-683.

Chan, Y.-F., Sam, I., & AbuBakar, S. (2010). Phylogenetic designation of enterovirus 71 genotypes and subgenotypes using complete genome sequences. *Infection, Genetics and Evolution*, *10*(3), 404-412.

Chang, L.-Y., King, C.-C., Hsu, K.-H., Ning, H.-C., Tsao, K.-C., Li, C.-C., Chen, P.-Y. (2002). Risk factors of enterovirus 71 infection and associated hand, foot, and mouth disease/herpangina in children during an epidemic in Taiwan. *Pediatrics*, *109*(6), e88-e88.

Chen, H.-F., Chang, M.-H., Chiang, B.-L., & Jeng, S.-T. (2006). Oral immunization of mice using transgenic tomato fruit expressing VP1 protein from enterovirus 71. *Vaccine*, *24*(15), 2944-2951.

Chen, H.-L., Huang, J.-Y., Chu, T.-W., Tsai, T.-C., Hung, C.-M., Lin, C.-C., Lin, M.-F. (2008). Expression of VP1 protein in the milk of transgenic mice: a potential oral vaccine protects against enterovirus 71 infection. *Vaccine*, *26*(23), 2882-2889.

Chen, Y., Li, C., He, D., Cheng, T., Ge, S., Shih, J. W.-K., Xia, N. (2013). Antigenic analysis of divergent genotypes human Enterovirus 71 viruses by a panel of neutralizing monoclonal antibodies: current genotyping of EV71 does not reflect their antigenicity. *Vaccine*, *31*(2), 425-430.

Chiu, C.-H., Chu, C., He, C.-C., & Lin, T.-Y. (2006). Protection of neonatal mice from lethal enterovirus 71 infection by maternal immunization with attenuated< i> Salmonella enterica</i> serovar Typhimurium expressing VP1 of enterovirus 71. *Microbes and infection*, *8*(7), 1671-1678.

Chong, P., Hsieh, S.-Y., Liu, C.-C., Chou, A.-H., Chang, J.-Y., Wu, S.-C., Klein, M. (2012). Production of EV71 vaccine candidates. *Hum Vaccin Immunother*, *8*(12), 1775-1783.

Chong, P., Liu, C. C., Chow, Y. H., Chou, A. H., & Klein, M. (2014). Review of enterovirus 71 vaccines. *Clinical Infectious Diseases*, ciu852.

Chumakov, M., Voroshilova, M., Shindarov, L., Lavrova, I., Gracheva, L., Koroleva, G., Gyurova, S. (1979). *Enterovirus 71* isolated from cases of epidemic poliomyelitis-like disease in Bulgaria. *Archives of virology, 60*(3-4), 329-340.

Chung, Y.-C., Ho, M.-S., Wu, J.-C., Chen, W.-J., Huang, J.-H., Chou, S.-T., & Hu, Y.-C. (2008). Immunization with virus-like particles of *enterovirus 71* elicits potent immune responses and protects mice against lethal challenge. *Vaccine*, 26(15), 1855-1862.

Chung, Y.-C., Huang, J.-H., Lai, C.-W., Sheng, H.-C., Shih, S.-R., Ho, M.-S., & Hu, Y.-C. (2006). Expression, purification and characterization of *enterovirus-71* virus-like particles. *World Journal of Gastroenterology, 12*(6), 921.

Cleland, J. L. (1999). Single-administration vaccines: controlled-release technology to mimic repeated immunizations. *Trends in biotechnology, 17*(1), 25-29.

Coleman, P. J., Shaw Jr, F. E., Serovich, J., Hadler, S. C., & Margolis, H. S. (1991). Intradermal hepatitis B vaccination in a large hospital employee population. *Vaccine, 9*(10), 723-727.

Craig, M. E., Howard, N. J., Silink, M., & Rawlinson, W. D. (2003). Reduced frequency of HLA DRB1* 03-DQB1* 02 in children with type 1 diabetes associated with *enterovirus* RNA. *Journal of Infectious Diseases*, 187(10), 1562-1570.

Davis, H. L., & McCluskie, M. J. (1999). DNA vaccines for viral diseases. *Microbes and Infection, 1*(1), 7-21.

De, W., Changwen, K., Wei, L., Monagin, C., Jin, Y., Cong, M, Jun, S. (2011). A large outbreak of hand, foot, and mouth disease caused by EV71 and CAV16 in Guangdong, China, 2009. *Archives of virology*, 156(6), 945-953.

Debing, Y., Jochmans, D., & Neyts, J. (2013). Intervention strategies for emerging viruses: use of antivirals. *Current opinion in virology*.

Di Martino A.., Sottinger M., Risbud M. V., Chitosan: A versatile biopolymer *Biomaterials*, (2005), 26, 5983–5990.

Ding, X., Shi, L., Liu, C., & Sun, B. (2013). A randomized comparison study of Aquacel Ag and Alginate Silver as skin graft donor site dressings. *Burns, 39*(8), 1547-1550.

Dodane V, Vilivalam VD. (1998), Pharmaceutical application of Chitosan. *PharmSci Technol Today*; 1:246-53.

Duclos, P. (2003). Safety of immunisation and adverse events following vaccination against hepatitis B. *Expert opinion on drug safety*, 2(3), 225-231.

Dutta, A. (2008). Epidemiology of poliomyelitis—options and update. *Vaccine, 26*(45), 5767-5773.

Foo, D. G. W., Alonso, S., Phoon, M. C., Ramachandran, N., Chow, V. T. K., & Poh, C. L. (2007). Identification of neutralizing linear epitopes from the VP1 capsid protein of Enterovirus 71 using synthetic peptides. *Virus research*, 125(1), 61-68.

Galindo-Rodriguez SA, Allemann E, Fessi H, Doelker E. (2005) Polymeric nanoparticles for oral delivery of drugs and vaccines: a critical evaluation of *in-vivo* studies. *Crit Rev Ther Drug Carrier Sys*; 22:419-64.

Gloudemans, A. K., Plantinga, M., Guilliams, M., Willart, M. A., Ozir-Fazalalikhan, A., Van Der Ham, A., . Hoogsteden, H. C. (2013). The mucosal adjuvant cholera toxin B instructs non-mucosal dendritic cells to promote IgA production via retinoic acid and TGF-β *PloS one, 8*(3), e59822.

Grassly, N. C. (2013). The final stages of the global eradication of poliomyelitis. *Philosophical Transactions of the Royal Society B: Biological Sciences, 368*(1623), 20120140.

Gupta, R. K., & Siber, G. R. (1995). Adjuvants for human vaccines-current status, problems and future prospects. *Vaccine, 13*(14), 1263-1276.

Haase, A. T. (2010). Targeting early infection to prevent HIV-1 mucosal transmission. *Nature, 464*(7286), 217-223.

Han, J.-F., Cao, R.-Y., Jiang, T., Yu, M., Liu, W., Tian, X., Qin, C.-F. (2011). Echovirus 30 in EV71-associated hand, foot and mouth disease outbreak, Guangxi, China. *Journal of Clinical Virology, 50*(4), 348-349.

Hayward, J. C., Gillespie, S. M., Kaplan, K. M., Packer, R., Pallansch, M., Plotkin, S., & Schonberger, L. B. (1989). Outbreak of poliomyelitis-like paralysis associated with enterovirus 71. *The Pediatric infectious disease journal, 8*(9), 611-615.

Holmgren, J., & Czerkinsky, C. (2005). Mucosal immunity and vaccines. *Nature medicine, 11*, S45-S53.

Huang, S.-C., Hsu, Y.-W., Wang, H.-C., Huang, S.-W., Kiang, D., Tsai, H.-P, Su, I.-J. (2008). Appearance of intratypic recombination of enterovirus 71 in Taiwan from 2002 to 2005. *Virus research*, 131(2), 250-259.

Huang, S.-W., Hsu, Y.-W., Smith, D. J., Kiang, D., Tsai, H.-P., Lin, K.-H., Wang, J.-R. (2009). Reemergence of enterovirus 71 in 2008 in Taiwan: dynamics of genetic and antigenic evolution from 1998 to 2008. *Journal of clinical microbiology, 47*(11), 3653-3662.

Illum L, Jabbal-Gill I, Hinchcliffe M. (2001) Chitosan as a novel nasal delivery system for vaccines. *Adv Drug Del Rev*; 51:81-96.

Janes KA, Calvo P, Alonso MJ. (2001) Polysaccharide colloidal particles as delivery systems for macromolecules. *Adv Drug Del Rev*; 47: 57-83.

Jiang, D., Premachandra, G. S., Johnston, C., & Hem, S. L. (2004). Structure and adsorption properties of commercial Calcium phosphate adjuvant. *Vaccine, 23*(5), 693-698.

Jin, M., Shan, J., Chen, Z., Guo, X., Shen, Z., Qiu, Z., Wang, X. (2013). Chlorine Dioxide Inactivation of Enterovirus 71 in Water and Its Impact on Genomic Targets. *Environmental science & technology, 47*(9), 4590-4597.

Jinfeng Xing, Liandong Deng, Anjie Dong, (2010), Chitosan/Alginate Nanoparticles Stabilized by Poloxamer for the Controlled Release of 5-Fluorouracil, *Journal Applied Poly. Sci* .,Vol. 117, 2354–2359.

Jordan, M., & Wurm, F. (2004). Transfection of adherent and suspended cells by Calcium phosphate. *Methods, 33*(2), 136-143.

Joyce, J. G., Krauss, I. J., Song, H. C., Opalka, D. W., Grimm, K. M., Nahas, D. D., Dudkin, V. Y. (2008). An oligosaccharide-based HIV-1 2G12 mimotope vaccine induces carbohydrate-specific antibodies that fail to neutralize HIV-1 virions. *Proceedings of the National Academy of Sciences, 105*(41), 15684-15689.

K Garg, N., Mangal, S., Khambete, H., K Sharma, P., & K Tyagi, R. (2010). Mucosal delivery of vaccines: role of mucoadhesive/biodegradable polymers. *Recent patents on drug delivery & formulation, 4*(2), 114-128.

Kew, O. M., Wright, P. F., Agol, V. I., Delpeyroux, F., Shimizu, H., Nathanson, N., & Pallansch, M. A. (2004). Circulating vaccine-derived polioviruses: current state of knowledge. *Bulletin of the World Health Organization, 82*(1), 16-23.

Kiener, T. K., Premanand, B., & Kwang, J. (2013). Immune responses to baculovirus-displayed enterovirus 71 VP1 antigen. *Expert review of vaccines, 12*(4), 357-364.

Kiparissides, C., & Kammona, O. (2013). Nanoscale carriers for targeted delivery of drugs and therapeutic biomolecules. The Canadian Journal of Chemical Engineering, 91(4), 638-651.

Ladet, S., David, L., Domard, A., (2008). Multi-membrane hydrogels. Nature (London,bU.K.) 452, 76–79.

Lakshman, L. R., Kumar, P., Nair, S. V., Nair, S. V., & Jayakumar, R. (2013). Chitosan Sponge Containing the Herb Coleus Plectranthus as a Wound Dressing. *Journal of Chitin and Chitosan Science, 1*(1), 13-20.

Larsen, G. R., Anderson, C. W., Dorner, A. J., Semler, B. L., & Wimmer, E. (1982). Cleavage sites within the poliovirus capsid protein precursors. Journal of virology, 41(1), 340-344.

Lehr CM, Bouwstra JA, Junginger HE. (1992) *In vitro* evaluation of mucoadhesive properties of Chitosan and some other nautral polymers. *Int J Pharm*; 78:43-8.

Liu, C.-C., Chou, A.-H., Lien, S.-P., Lin, H.-Y., Liu, S.-J., Chang, J.-Y, Chang, K. H.-W. (2011). Identification and characterization of a cross-neutralization epitope of Enterovirus 71. *Vaccine*, 29(26), 4362-4372.

Li, L., He, Y., Yang, H., Zhu, J., Xu, X., Dong, J., Jin, Q. (2005). Genetic characteristics of human enterovirus 71 and coxsackievirus A16 circulating from 1999 to 2004 in Shenzhen, People's Republic of China. *Journal of clinical microbiology*, *43*(8), 3835-3839.

Li, J., Huo, X., Dai, Y., Yang, Z., Lei, Y., Jiang, Y.,Zhan, F. (2012). Evidences for intertypic and intratypic recombinant events in EV71 of hand, foot and mouth disease during an epidemic in Hubei Province, China, 2011. *Virus Research*.

Li, JX., Meng, F., Liang, Z., Mao, Q., & Zhu, F. (2013). How to understand the efficacy measurements for enterovirus type 71 vaccine? *Human vaccines & immunotherapeutics*, *10*(3).

Li, P., Dai, Y.-N., Zhang, J.-P., Wang, A.-Q., & Wei, Q. (2008). Chitosan-Alginate nanoparticles as a novel drug delivery system for nifedipine. *International journal of biomedical science: IJBS*, *4*(3), 221.

LI, W., & YE, D.-Q. (2008). Progression on the study of EV71 complicating neurogenic pulmonary edema [J]. *Chinese Journal of Disease Control & Prevention*, *3*, 004.

Li, Y.-P., Liang, Z.-L., Xia, J.-L., Wu, J.-Y., Wang, L., Song, L.-F., Hu, Y.-S. (2014). Immunogenicity, Safety, and Immune Persistence of a Novel Inactivated Human Enterovirus 71 Vaccine: A Phase II, Randomized, Double-Blind, Placebo-Controlled Trial. *Journal of Infectious Diseases*, *209*(1), 46-55.

Liang, Z.-L., Mao, Q.-Y., Wang, Y.-P., Zhu, F.-C., Li, J.-X., Yao, X., Wang, J.-Z. (2013). Progress on the research and development of inactivated EV71 whole-virus vaccines. *Hum Vaccin Immunother*, *9*(8), 1701-1705.

Liming Hu,Yun Sun,Yan Wu (2013) Advances in Chitosan-based drug delivery vehicles , *Nanoscale*, 5, 3103-3111, DOI: 10.1039/C3NR00338H.

Lin, K. H., Hwang, K. P., Ke, G. M., Wang, C. F., Ke, L. Y., Hsu, Y. T., Chen, H. L. (2006). Evolution of EV71 genogroup in Taiwan from 1998 to 2005: an emerging of subgenogroup C4 of EV71. *Journal of medical virology*, *78*(2), 254-262\

Liu, L., Zhang, Y., Wang, J., Zhao, H., Jiang, L., Che, Y., Huang, T. (2013). Study of the Integrated Immune Response Induced by an Inactivated EV71 Vaccine. *PloS one*, *8*(1), e54451.

Li, R., Liu, L., Mo, Z., Wang, X., Xia, J., Liang, Z., Wang, J. (2014). An inactivated enterovirus 71 vaccine in healthy children. *New England Journal of Medicine*, *370*(9), 829-837.

Longer MA, Cheng HS, Robinson JR. (1985) Bioadhesive polymers as platforms for oral controlled drug delivery III: oral delivery of chlorothiazide using a bioadhesive polymer. J Pharm Sci; 74: 406-11.

Lum, L., Wong, K., Lam, S., Chua, K., Goh, A., Lim, W., Lambert, M. (1998). Fatal enterovirus 71 encephalomyelitis. *The Journal of pediatrics*, *133*(6), 795-798.

Lawson, L. B., Norton, E. B., & Clements, J. D. (2011). Defending the mucosa: adjuvant and carrier formulations for mucosal immunity. *Current opinion in immunology*, *23*(3), 414-420.

M. Dasha, F. Chiellini a, R.M. Ottenbriteb, E. Chiellini, (2011) Chitosan—A versatile semi-synthetic polymer in biomedical applications, *Progress in Polymer Science* 36, 981–1014.

Ma, S.-h., Liu, J.-s., Wang, J.-j., Shi, H.-j., Yang, H.-j., Chen, J.-y., Li, Q.-h. (2009). Genetic analysis of the VP1 region of human enterovirus 71 strains isolated in Fuyang, China, during 2008. *Virologica Sinica*, *24*(3), 162-170.

Ma, Z., & Lim, L.-Y. (2003). Uptake of Chitosan and associated insulin in Caco-2 cell monolayers: a comparison between Chitosan molecules and Chitosan nanoparticles. *Pharmaceutical Research*, *20*(11), 1812-1819.

Mao, Q., Dong, C., Li, X., Gao, Q., Guo, Z., Yao, X., Xu, M. (2012). Comparative analysis of the immunogenicity and protective effects of inactivated EV71 vaccines in mice. *PloS one*, *7*(9), e46043.

Mao, S., Shuai, X., Unger, F., Simon, M., Bi, D., & Kissel, T. (2004). The depolymerization of Chitosan: effects on physicochemical and biological properties. *International journal of pharmaceutics*, *281*(1), 45-54.

Martín, J., Samoilovich, E., Dunn, G., Lackenby, A., Feldman, E., Heath, A., Minor, P. D. (2002). Isolation of an intertypic poliovirus capsid recombinant from a child with vaccine-associated paralytic poliomyelitis. *Journal of virology*, *76*(21), 10921-10928.

McGhee, J. R., Mestecky, J., Dertzbaugh, M. T., Eldridge, J. H., Hirasawa, M., & Kiyono, H. (1992). The mucosal immune system: from fundamental concepts to vaccine development. *Vaccine*, *10*(2), 75-88.

McGhee, J. R., & Kiyono, H. (1993). New perspectives in vaccine development: mucosal immunity to infections. *Infectious agents and disease*, *2*(2), 55-73.

McMinn, P. C. (2002). An overview of the evolution of enterovirus 71 and its clinical and public health significance. *FEMS microbiology reviews*, 26(1), 91-107.

Meng, T., Kolpe, A. B., Kiener, T. K., Chow, V. T., & Kwang, J. (2011). Display of VP1 on the surface of baculovirus and its immunogenicity against heterologous human enterovirus 71 strains in mice. *PLoS One*, *6*(7), e21757.

Modlin, J. F. (2004). Poliomyelitis in the United States: the final chapter? *Jama, 292*(14), 1749-1751.

Morris, W., Steinhoff, M. C., & Russell, P. K. (1994). Potential of polymer microencapsulation technology for vaccine innovation. *Vaccine, 12*(1), 5-11.

Moszynski, P. (2013). Polio is re-emerging in areas previously considered polio free. *BMJ: British Medical Journal, 347*.

Neutra, M. R., & Kozlowski, P. A. (2006). Mucosal vaccines: the promise and the challenge. *Nature Reviews Immunology, 6*(2), 148-158.

Nguyen, D. N., Green, J. J., Chan, J. M., Langer, R., & Anderson, D. G. (2009). Polymeric materials for gene delivery and DNA vaccination. *Advanced Materials, 21*(8), 847-867.

O'Hagan, D. T. (1998). Microparticles and polymers for the mucosal delivery of vaccines. *Advanced drug delivery reviews, 34*(2), 305-320.

O'Hagan, D. T., Ott, G. S., & Van Nest, G. (1997). Recent advances in vaccine adjuvants: the development of MF59 emulsion and polymeric microparticles. *Molecular medicine today, 3*(2), 69-75.

O'Meara, S., St James, M. M., & Jull, A. (2013). Alginate dressings for venous leg ulcers. *Cochrane Database Syst Rev, 4*.

Onorato, I. M., Modlin, J. F., McBean, A. M., Thoms, M. L., Losonsky, G. A., & Bernier, R. H. (1991). Mucosal immunity induced by enhanced-potency inactivated and oral polio vaccines. *Journal of infectious diseases, 163*(1), 1-6.

Pal, K., Behera, B., Roy, S., Sekhar Ray, S., & Thakur, G. (2013). Chitosan based delivery systems on a length scale: nano to macro. *Soft Materials, 11*(2), 125-142.

Petrovsky, N., & Aguilar, J. C. (2004). Vaccine adjuvants: current state and future trends. *Immunology and cell biology, 82*(5), 488-496.

Phuektes, P. (2009). *Development of a reverse genetic system for Human enterovirus 71 (HEV71) and the molecular basis of its growth phenotype and adaptation to mice*. Murdoch University.

Premanand, B., Prabakaran, M., Kiener, T. K., & Kwang, J. (2013). Recombinant baculovirus associated with bilosomes as an oral vaccine candidate against HEV71 infection in mice. *PloS one, 8*(2), e55536.

Prevots, D. R., Sutter, R. W., Strebel, P. M., Weibel, R. E., & Cochi, S. L. (1994). Completeness of reporting for paralytic poliomyelitis, United States, 1980 through 1991: implications for estimating the risk of vaccine-associated disease. *Archives of pediatrics & adolescent medicine, 148*(5), 479.

Ramachandran R, Paul W, Sharma CP. (2009) Synthesis and characterization of PEGylated Calcium phosphate nanoparticles for oral insulin delivery. *J Biomed Mater Res B Appl Biomate*r.; 88(1) 41-8.

Rappuoli, R. (1997). Current developments in new vaccines for adolescents. *Biologicals*, *25*(2), 159-163.

Rawat, M. Singh, D. Saraf, S. *et al*. (2008). Development and *in vitro* evaluation of Alginate gel-encapsulated, Chitosan-coated ceramic nanocores for oral delivery enzyme. *Drug Dev. Ind. Pharm*. 34: 181-188.

Relyveld, E.H., (1986). Preparation and use of Calcium phosphate adsorbed vaccines. *Dev. Biol. Stand*. 65, 131–136.

Ritger PL, Peppas NA. (1987) A simple equation for description of solute release I, Fickian and non-Fickian release from non-swellable devices in the form of slabs, spheres, cylinders, or discs. *J Controlled Release*, 5:23-36.

Ryan, E. J., Daly, L. M., & Mills, K. H. (2001). Immunomodulators and delivery systems for vaccination by mucosal routes. *TRENDS in Biotechnology*, *19*(8), 293-304.

Sahasathian, T., Praphairaksit, N., & Muangsin, N. (2010). Mucoadhesive and floating Chitosan-coated Alginate beads for the controlled gastric release of amoxicillin. *Archives of pharmacal research*, *33*(6), 889-899.

Sahoo, S. K., & Labhasetwar, V. (2003). Nanotech approaches to drug delivery and imaging. *Drug discovery today*, *8*(24), 1112-1120.

Saluja, V., Amorij, J. P., van Roosmalen, M. L., Leenhouts, K., Huckriede, A., Hinrichs, W. L., & Frijlink, H. W. (2010). Intranasal delivery of influenza subunit vaccine formulated with GEM particles as an adjuvant. *The AAPS journal*, *12*(2), 109-116.

Sarma, N. (2013). Relapse of hand foot and mouth disease: Are we at more risk? *Indian journal of dermatology*, *58*(1), 78.

Sarmento B, Ferreira DC, Jorgensen L, Vande Weert M. (2007) Probing insulin's secondary structure after entrapment into Alginate Chitosan nanoparticles. *Eur J Pharm Biopharm* 65:10-7.

Scherließ R, *et al*, (2013) *in-vivo* evaluation of Chitosan as an adjuvant in subcutaneous vaccine formulations. *Vaccine*, 31(42):4812-4819.

Shahiwala, A., Vyas, T. K., & Amiji, M. M. (2007). Nanocarriers for systemic and mucosal vaccine delivery. *Recent patents on drug delivery & formulation*, *1*(1), 1-9.

Shenyu, W., Jingxin, L., Zhenglun, L., Xiuling, L., Qunying, M., Fanyue, M, Qinghua, C. (2014). A booster dose of an inactivated enterovirus 71 vaccine in Chinese young children: a randomized, double-blind, placebo-controlled clinical trial. *Journal of Infectious Diseases*, jiu113.

Shi, W., Li, K., Ji, Y., Jiang, Q., Shi, M., & Mi, Z. (2011). Development and evaluation of reverse transcription-loop-mediated isothermal amplification assay for rapid detection of enterovirus 71. *BMC infectious diseases*, 11(1), 197.

Singh, M., Carlson, J. R., Briones, M., Ugozzoli, M., Kazzaz, J., Barackman, J., O'Hagan, D. (1998). A comparison of biodegradable microparticles and MF59 as systemic adjuvants for recombinant gD from HSV-2. *Vaccine, 16*(19), 1822-1827.

Singh, M., Briones, M., Ott, G., & O'Hagan, D. (2000). Cationic microparticles: a potent delivery system for DNA vaccines. *Proceedings of the National Academy of Sciences, 97*(2), 811-816.

Singh, M., & O'Hagan, D. (1999). Advances in vaccine adjuvants. *Nature biotechnology, 17*(11), 1075-1081.

Singh, S., Chow, V. T., Phoon, M., Chan, K., & Poh, C. L. (2002). Direct detection of enterovirus 71 (EV71) in clinical specimens from a hand, foot, and mouth disease outbreak in Singapore by reverse transcription-PCR with universal enterovirus and EV71-specific primers. *Journal of clinical microbiology, 40*(8), 2823-2827.

Solomon, T., Lewthwaite, P., Perera, D., Cardosa, M. J., McMinn, P., & Ooi, M. H. (2010). Virology, epidemiology, pathogenesis, and control of enterovirus 71. *The Lancet infectious diseases, 10*(11), 778-790.

Svitlana Chernousova, Jan Klesing, Nadiia Soklakova and Matthias Epple, (2013). A genetically active nano-Calcium phosphate paste for bone substitution, encoding the formation of BMP-7 and VEGF-A, *RSC Adv*, 3, 11155-11161 DOI: 10.1039/C3RA23450A.

Takada, A., Matsushita, S., Ninomiya, A., Kawaoka, Y., & Kida, H. (2003). Intranasal immunization with formalin-inactivated virus vaccine induces a broad spectrum of heterosubtypic immunity against influenza A virus infection in mice. *Vaccine, 21*(23), 3212-3218.

Takeuchi, H., Yamamoto, H., & Kawashima, Y. (2001). Mucoadhesive nanoparticulate systems for peptide drug delivery. *Advanced Drug Delivery Reviews, 47*(1), 39-54.

Thibaut, H. J., Leyssen, P., Puerstinger, G., Muigg, A., Neyts, J., & De Palma, A. M. (2011). Towards the design of combination therapy for the treatment of enterovirus infections. *Antiviral research, 90*(3), 213-217.

Tiselius A. Hjerte´n S., Levin, O, (1956) Protein chromatography on Calcium Phosphate columns. *Archives of biochemistry and biophysics*, *65*(1), 132-155

Trapani, A., Di Gioia, S., Ditaranto, N., Cioffi, N., Goycoolea, F. M., Carbone, A., Alonso, M. J. (2013). Systemic heparin delivery by the pulmonary route using Chitosan and glycol Chitosan nanoparticles. *International journal of pharmaceutics*.

Van der Lubben, I., Verhoef, J., Borchard, G., & Junginger, H. (2001). Chitosan for mucosal vaccination. *Advanced drug delivery reviews*, *52*(2), 139-144

Varma, N. R. S., Toosa, H., Foo, H. L., Alitheen, N. B. M., Nor Shamsudin, M., Arbab, A. S., Abdul Rahim, R. (2013). Display of the Viral Epitopes on Lactococcus lactis: A Model for Food Grade Vaccine against EV71. *Biotechnology research international, 2013*.

Vladimir Voynov and Justin A. Caravella (2013). Therapeutic Proteins: Methods and Protocols, Methods in Molecular Biology, vol. 899, DOI 10.1007/978-1-61779-921-1_28, © Springer Science+Business Media,

Vogel, F. R. (2000). Improving vaccine performance with adjuvants. *Clinical Infectious Diseases*, *30*(Supplement 3), S266-S270.

Wang, S.-M., & Liu, C.-C. (2014). Update of enterovirus 71 infection: epidemiology, pathogenesis and vaccine. *Expert review of anti-infective therapy*, *12*(4), 447-456.

Wang, X., Peng, W., Ren, J., Hu, Z., Xu, J., Lou, Z., Porta, C. (2012). A sensor-adaptor mechanism for enterovirus uncoating from structures of EV71. *Nature structural & molecular biology*, *19*(4), 424-429.

Wang, Y.-p., LI, M.-y., Gao, F., Shao, J., Mao, Q.-y., YAO, X., Liang, Z.-l. (2012). Effect of aluminum hydroxide adjuvant on cellular immune response induced with inactivated enterovirus 71 vaccine in mice [J]. *Chinese Journal of Biologicals*, *8*, 004.

Watkins, R. E., Martin, P. A. J., Kelly, H., Madin, B., & Watson, C. (2009). An evaluation of the sensitivity of acute flaccid paralysis surveillance for poliovirus infection in Australia. *BMC infectious diseases*, *9*(1), 162.

Wong, S., Yip, C., Lau, S., & Yuen, K. (2010). Human enterovirus 71 and hand, foot and mouth disease. *Epidemiology and infection*, *138*(8), 1071-1089.

Wu, C.-N., Lin, Y.-C., Fann, C., Liao, N.-S., Shih, S.-R., & Ho, M.-S. (2001). Protection against lethal enterovirus 71 infection in newborn mice by passive immunization with subunit VP1 vaccines and inactivated virus. *Vaccine*, 20(5), 895-904.

Xu, J., Qian, Y., Wang, S., Serrano, J. M. G., Li, W., Huang, Z., & Lu, S. (2010). EV71: an emerging infectious disease vaccine target in the Far East? *Vaccine, 28*(20), 3516-3521.

Xu, Y., & Du, Y. (2003). Effect of molecular structure of Chitosan on protein delivery properties of Chitosan nanoparticles. *International journal of pharmaceutics, 250*(1), 215-226.

Yan, J. J., Su, I. J., Chen, P. F., Liu, C. C., Yu, C. K., & Wang, J. R. (2001). Complete genome analysis of enterovirus 71 isolated from an outbreak in Taiwan and rapid identification of enterovirus 71 and coxsackievirus A16 by RT-PCR. *Journal of medical virology, 65*(2), 331-339.

Yan, X.-F., Gao, S., Xia, J.-F., Ye, R., Yu, H., & Long, J.-E. (2012). Epidemic characteristics of hand, foot, and mouth disease in Shanghai from 2009 to 2010: Enterovirus 71 subgenotype C4 as the primary causative agent and a high incidence of mixed infections with coxsackievirus A16. *Scandinavian Journal of Infectious Diseases, 44*(4), 297-305.

Yang, C., Deng, C., Wan, J., Zhu, L., & Leng, Q. (2011). Neutralizing antibody response in the patients with hand, foot and mouth disease to enterovirus 71 and its clinical implications. *Virol J*, 8(1), 306-311.

Yang, F., Ren, L., Xiong, Z., Li, J., Xiao, Y., Zhao, R., Wang, J. (2009). Enterovirus 71 outbreak in the People's Republic of China in 2008. *Journal of clinical microbiology*, 47(7), 2351-2352.

Yang, T., Xu, G., Dong, H., Ye, M., & He, T. (2012). A case–control study of risk factors for severe hand–foot–mouth disease among children in Ningbo, China, 2010–2011. *European journal of pediatrics, 171*(9), 1359-1364.

Yao, X., Mao, Q.-y., & Liang, Z.-l. (2011). Progress in Research on Enterovirus 71 Vaccine [J]. *Chinese Journal of Biologicals, 2*, 030.

Yu, C.-K., Chen, C.-C., Chen, C.-L., Wang, J.-R., Liu, C.-C., Yan, J.-J., & Su, I.-J. (2000). Neutralizing antibody provided protection against enterovirus type 71 lethal challenge in neonatal mice. *Journal of biomedical science, 7*(6), 523-528.

Yu, C.-K., Liu, C.-C., Wang, S.-M., Lei, H.-Y., Su, I.-J., Wu, T.-C., Wang, J.-R. (2007). Immunity to Avirulent Enterovirus 71 and. *J. Virol, 81*(19), 10310.

Yu, Z., Huang, Z., Sao, C., Huang, Y., Zhang, F., Ma, G., Zeng, W. (2013). Oral immunization of mice using Bifidobacterium longum expressing VP1 protein from enterovirus 71. *Archives of virology*, *158*(5), 1071-1077.

Zhang, D., Lu, J., & Lu, J. (2010). Enterovirus 71 vaccine: close but still far. *International Journal of Infectious Diseases, 14*(9), e739-e743.

Zhang, F. (2013). Progress on the research and development of EV71 vaccine. *Journal of Applied Virology, 2*(4), 1-6.

Zhang, Y., Zhu, Z., Yang, W., Ren, J., Tan, X., Wang, Y., Cui, A. (2010). An emerging recombinant human enterovirus 71 responsible for the 2008 outbreak of hand foot and mouth disease in Fuyang city of China. *Virology journal, 7*(1), 94.

Zhou, X., Liu, B., Yu, X., Zha, X., Zhang, X., Chen, Y., Chen, Y. (2007). Controlled release of PEI/DNA complexes from mannose-bearing Chitosan microspheres as a potent delivery system to enhance immune response to HBV DNA vaccine. *Journal of Controlled Release, 121*(3), 200-207.

Zhu, F.-C., Meng, F.-Y., Li, J.-X., Li, X.-L., Mao, Q.-Y., Tao, H., Chen, Q.-H. (2013). Efficacy, safety, and immunology of an inactivated alum-adjuvant enterovirus 71 vaccine in children in China: a multicentre, randomised, double-blind, placebo-controlled, phase 3 trial. *The Lancet*.